[CONTRIBUTION FROM THE LABORATORY OF PHYSICAL CHEMISTRY OF THE UNIVERSITY OF UPSALA]

A NEW METHOD FOR THE DETERMINATION OF THE MOLECULAR WEIGHT OF THE PROTEINS

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The lack of a reliable method for the determination of the molecular weights of substances possessing a very complicated structure has been a serious obstacle in the progress of our knowledge of the chemistry of the proteins. In the present paper such a method will be proposed and its use will be illustrated by a few preliminary measurements on hemoglobin.

The Method

The procedure is based upon the fact already pointed out by one of us[1] that it should be possible to determine the mass of heavy molecules by measuring the sedimentation equilibrium in the ultracentrifuge.

When a solution is centrifuged in a closed cell for a sufficiently long time a state of equilibrium is finally reached when sedimentation and diffusion balance each other. This means that during the time dt the quantity ds of the solute that is driven by centrifugal force through the unit surface in the direction of the periphery is the same as that which is wandering in the direction towards the center of rotation by virtue of diffusion.

For the sedimentation we have

$$ds = c\omega^2 \times M\,(1 - V\rho).1/F.dt \qquad (1)$$

and for the diffusion

$$ds = -RT.dc/dx.1/F.dt \qquad (2)$$

where R is the gas constant, T the absolute temperature, M the molecular weight of solute, F the frictional force exerted upon a mole of solute, V the partial specific volume of solute, ρ the density of solvent, c the concentration, ω the angular velocity of centrifuge, and x the distance from the center of rotation (positive in the direction toward center of rotation).

Equating and eliminating we get

$$dc/c = -\frac{M(1 - V\rho)\omega^2 x dx}{RT} \qquad (3)$$

After integrating this expression between the points x_1 and x_2 we finally have for the molecular weight

$$M = \frac{2\,RT\,ln\,(c_1/c_2)}{\omega^2\,(1 - V\rho)\,(x_1 - x_2)\,(x_1 + x_2)} \qquad (4)$$

To determine the molecular weight it is therefore necessary only to measure the relation between the concentration of the solution at two points situated x_1 and x_2 cm. from the center of rotation and to know the temperature, the speed of the centrifuge, the partial specific volume of the solute and the density of the solvent.

[1] Svedberg, *Kolloid-Z., Zsigmondy-Festschrift*, **1925**, p. 53.

The formula holds only for dilute solutions. In the case of concentrated solutions the formula for the molecular weight will include the expression for the partial specific free energy of the solute.[2] When the solute is electrolytically dissociated the formula for the molecular weight has to be modified.[3] We refrain from giving the theory of these cases as the proteins can be studied in sufficiently dilute solution and because the dissociation of the proteins is small at least at the iso-electric point.

The ultracentrifuge used in these measurements has recently been described in THIS JOURNAL.[4] A small quantity of the solution to be studied, from 0.01 to 0.25 cc., is enclosed in a glass or quartz cell possessing plane parallel walls and rotated at constant temperature in a special centrifuge which permits photographing the solution when exposed to a

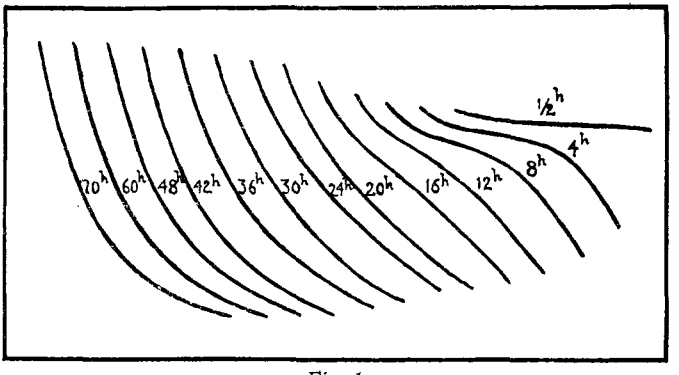

Fig. 1.

centrifugal force up to 5000 times the force of gravity. After equilibrium has been reached and the cell photographed a series of solutions of different concentrations is photographed in the same cell shortly after starting the centrifuge, that is, before any marked sedimentation has set in. The plate carrying the photograph of the solution on its way to sedimentation equilibrium and after reaching it, as well as the scale of different concentrated solutions is then recorded by means of a self-registering microphotometer. From the records thus obtained the curve giving the relation between concentration of the solution and distance from the center of rotation can easily be constructed. In Fig. 1 curves traced from the microphotometric records are given illustrating the progress of centrifuging of a column of a 2.0% carbonmonoxide-hemoglobin solution 0.8 cm. long at a speed of 10,240 r.p.m. The abscissas are the distances from center of rotation and the ordinates are the deflections of the galvanometer

[2] Compare Lewis and Randall, "Thermodynamics," McGraw-Hill Book Co., New York, 1923, p. 244.

[3] Ref. 1, p. 63.

[4] Svedberg and Rinde, THIS JOURNAL, 46, 2677 (1924).

of the microphotometer—a quantity nearly proportional to the concentration of the solution. Each curve is displaced 1 cm. to the left of the preceding one in order to avoid confusion. Fig. 2 gives an enlarged photograph of the solution after sedimentation equilibrium has been reached.

Fig. 2.

The cells used in the present work differ somewhat from those previously described.

Each cell is made up of three circular glass or quartz plates 2.0 cm. in diameter cemented together with de Khotinsky cement and surrounded by a brass ring (Fig. 3). In the middle plate a sectorial aperture of 5° angle and 5.2cm. radius has been cut out and in this cavity the solution is placed. In most of the experiments on hemoglobin the thickness of the layer of solution was 0.145 cm., but in some cases it was 0.98 cm. Evaporation from the solution is one of the most serious sources of error in experiments of this kind because of the eddy currents in the solution to which it gives rise. It was found that the best means of completely preventing evaporation is to cover the solution with a layer of liquid paraffin. The cell holder is a brass ring taking two pairs of cells of different thickness (Fig. 4). Two ring-shaped brass plates (not shown in the figure) of the same outline as the cell holder and provided with four sectorial apertures are screwed to the cell holder clamping the cells together firmly. An ebonite plate with only one sectorial aperture cuts off all the light except that from one of the cells.[5]

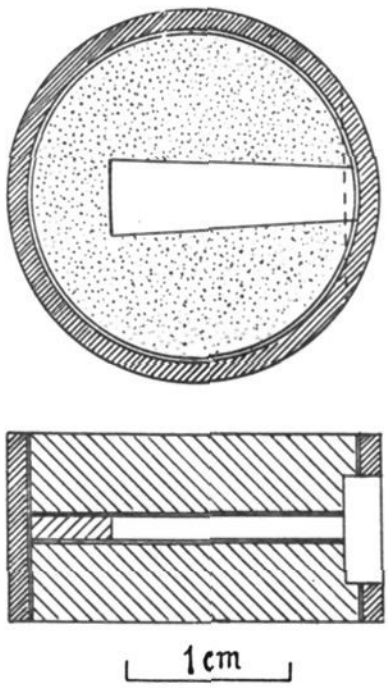

Fig. 3.

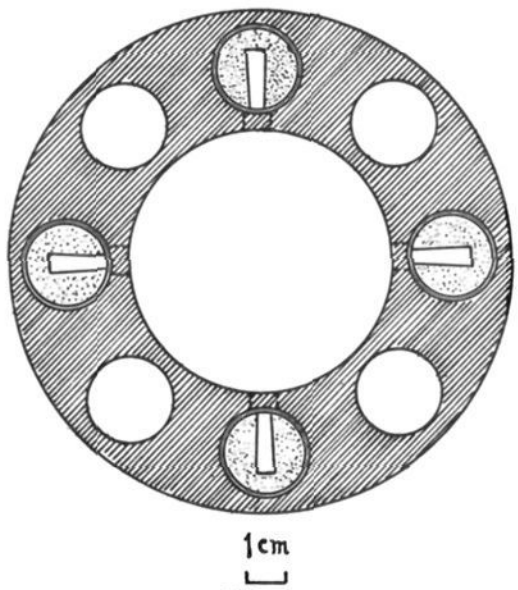

Fig. 4.

The time of centrifuging necessary for reaching sedimentation equilibrium was found to be about six hours per 0.1 cm. length of column of solution in the case of hemoglobin. Usually a length of about 0.5 cm. was chosen which requires 30 hours of centrifuging. The magnification of the

[5] We are indebted to Mr. Alf Lysholm of the Ljungström Steam-Turbin Co., Stockholm, for valuable suggestions with regard to the construction of the cells.

microphotometer was 10 and the photographs of the column of solution were taken in the scale 1:1 so that an abscissa of 0.1 cm. in the photograph corresponded to 1.0 cm. in the microphotometric curve. In the computation of the molecular weight from the curve giving the relation between concentration and distance from the center of rotation the difference $x_1 - x_2$ of Formula 4, was taken equal to 0.05 cm. A length of column of solution equal to 0.5 cm., therefore, gives 10 values of the molecular weight. If the solute studied is composed of molecules of equal mass these values should be constant. If it contains molecules of different mass, that is, if the solute is partly associated, the values of the molecular weight should decrease from the bottom of the cell upwards. As has already been shown by one of us[6] it should be possible to calculate the distribution curve for the different aggregates from an analysis of the concentration curve furnished by a centrifuging experiment.

For the study of such proteins which have absorption in the visible spectrum, for example, hemoglobin and phytoerythrin, a suitable incandescent lamp eventually combined with light filters is used as light source. The lamp has to be fed by accumulators and the voltage must be carefully controlled. In the case of substances possessing absorption only in the short-waved ultraviolet region, such as serum albumin, egg albumin, etc., a quartz mercury lamp with filters of gaseous chlorine and bromine is used. In the latter case the optical parts of the centrifuge must of course be made of quartz and fluorite. This technique has been developed using egg albumin as test material. Measurements on colorless proteins will be communicated later.

Measurements on Hemoglobin

The eminent physiological importance of hemoglobin has made the determination of its molecular weight, which enters as a factor in the formula for the respiratory power of the blood,[7] an urgent task. Moreover, the fact that a minimum value for the molecular weight, namely, 16,700, can be computed from the iron content, makes the determination of its actual molecular weight a very attractive problem. The ordinary methods based upon freezing-point, boiling-point and vapor-pressure determinations of course fail for a substance of such high molecular weight and the previous attempts to measure the molecular weight of hemoglobin all deal with the measurement of osmotic pressure against a semi-permeable membrane. Hüfner and Gansser[8] in their classical pioneer work arrived at a value for the molecular weight of oxyhemoglobin in a solution free from electrolytes very close to the minimum value 16,700. Reid[9] found

[6] Ref. 1, p. 61.

[7] Barcroft, "The Respiratory Function of the Blood," Cambridge Univ. Press, 1914.

[8] Hüfner and Gansser, *Arch. Anat. Physiol.*, **1907**, p. 209.

[9] Reid, *J. Physiol.*, **33**, 12 (1905–1906).

under the same conditions values which are about three times the minimum weight. In the presence of electrolytes Roaf[10] and Wilson[11] found values for the molecular weight varying from a fraction of the minimum weight to a high multiple of it. These rather conflicting results are probably due partly to the difficulty of measuring osmotic pressures against semi-permeable membranes and partly to the disturbing action of the Donnan effect. These difficulties make it highly desirable that some other more reliable method should be tried for the determination of the molecular weight of hemoglobin.

Our first experiments were made with dialyzed oxyhemoglobin in pure aqueous solution and in 1% potassium chloride solution. They gave for the molecular weight of hemoglobin values which were between three and four times the minimum value of 16,700. No marked influence of the potassium chloride was observed. Because of the instability of the oxy-hemoglobin (formation of met-hemoglobin) the following measurements were carried out on carbonmonoxide-hemoglobin and met-hemoglobin.

The solutions of carbonmonoxide-hemoglobin were prepared in the following way. Horse blood was defibrinated by shaking with pearls of Jena glass in a Jena-glass flask and saturated with carbon monoxide. The corpuscles were separated from the serum by centrifuging, washed seven times with 1.5% sodium chloride solution and dialyzed for 14 days in collodion bags immersed in flowing distilled water at 0°, saturated with toluene. The contents of the bags were centrifuged, a 10% stock solution prepared and again saturated with carbon monoxide. The conductivity at a dilution of 2% was 6×10^{-4} mhos.

Two series of determinations of the partial specific volume of carbon-monoxide–hemoglobin by means of pycnometric measurements at 20.0° were carried out on this material between the concentrations 2 and 10%. It was found that the partial specific volume varies but little with concentration. For dilute solutions the value 0.749 was adopted.

The met-hemoglobin solutions were prepared in the following way. Horse blood was defibrinated and the corpuscles were washed and dialyzed for 14 days as described above. The solution of oxyhemoglobin thus obtained was stored for some time at 0° and then treated with potassium ferricyanide until the oxyhemoglobin bands could no longer be seen. The solution was dialyzed for 22 days at 0° and filtered. The conductivity was 1×10^{-4} mhos in 2% solution.

A determination of the partial specific volume gave the value 0.747 which is identical with the value for carbonmonoxide-hemoglobin within the limits of experimental error.

The centrifuging experiments were carried out on solutions made by

[10] Roaf, *Quart. J. Exptl. Physiol.*, **3**, 75 (1910).

[11] Wilson, *Biochem. J.*, **17**, 59 (1923).

diluting these stock solutions and on samples further purified by means of Pauli's method of electrodialysis.[12] A number of determinations with the solution of carbonmonoxide-hemoglobin were carried out in order to show whether or not the values of the molecular weight found were dependent on the experimental conditions. Thus, measurements were made

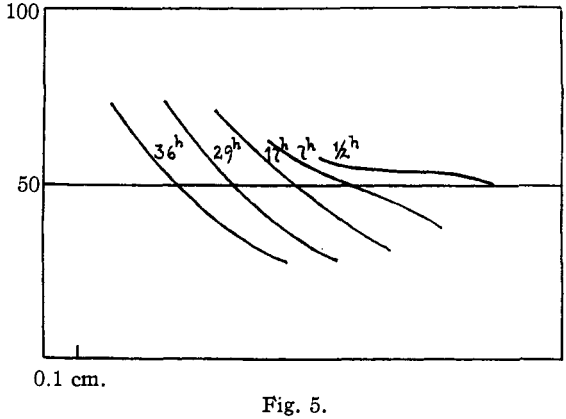

Fig. 5.

on solutions containing 0.5, 1,0, 2.0, 3.0 g. of carbonmonoxide-hemoglobin per 100 cc. of solution. The values of the molecular weight were constant within the limits of experimental error. The length of the column of solution was varied from 0.25 to 0.8 cm. without any marked influence upon the molecular weight. The same molecular weight was found when the speed of the centrifuge was 7200, 8700, and 10,000 r.p.m. It was further found that practically the same equilibrium was arrived at when the speed was raised from zero to 7200 r.p.m. and when it was lowered from 10,000 to 7200 r.p.m. In the latter case the comparatively high fall of concentration with distance from the center of rotation decreased by virtue of diffusion until the same state was reached as by direct centrifuging at 7200 r.p.m. In some of these experiments the centrifuging of a sample of solution was carried on for a week without interruption, but there is always some danger of changes taking place in the substance studied

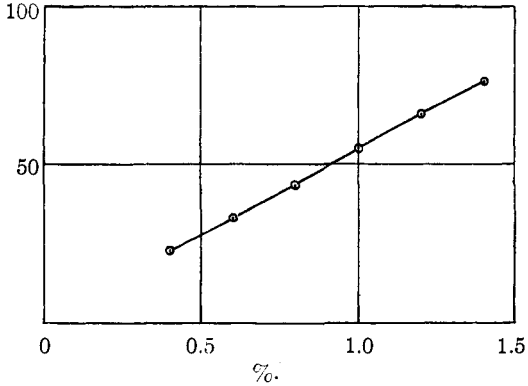

Fig. 6.

[12] Pauli, *Biochem. Z.*, **152**, 355 (1924)

during so long a time. Therefore as a rule longer experiments than 40 hours were avoided.

Four series of determinations, two on carbonmonoxide-hemoglobin solutions and two on met-hemoglobin, are given below.[13] One of the series on carbonmonoxide-hemoglobin is made on a practically salt-free electro-dialyzed solution (conductivity 5.0×10^{-4} mhos in a 2% solution), the other on a sample of the same solution with the addition of sodium chloride up to 0.1% (conductivity 1.8×10^{-3} mhos in a 2% solution). One of the determinations on met-hemoglobin refers to a solution purified only by ordinary dialysis (conductivity 1.1×10^{-4} mhos in a 2% solution), the other to a sample of the same solution further purified by electro-dialysis (conductivity 3.1×10^{-5} mhos in a 2% solution). All of these determinations give practically the same value for the molecular weight, namely, $4 \times 16{,}700$. The technique of the method is not yet refined enough to permit us to decide whether all the molecules in a hemoglobin solution are composed of four groups of the weight 16,700 or whether there is an equilibrium between molecules in various states of aggregation—an equilibrium which might be regarded as dependent upon concentration, salt content, etc. There is a circumstance, however, strongly

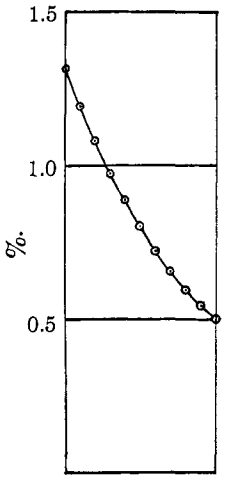

Fig. 7.

TABLE I

EXPERIMENTS

1. Solution of carbonmonoxide-hemoglobin containing 1.0 g. per 100 cc. at the start; conductivity, 5.0×10^{-5} mhos in a 2.0% solution; $V = 0.749$; $T = 293.3°$; length of column of solution, 0.6 cm.; thickness of column of solution, 0.145 cm.; speed, 8708 r.p.m. ($\omega = 290.3\pi$); time of centrifuging, 39 hours; light source, incandescent lamp with white bulb; light filter, Ilford three-color green; aperture of photographic lens, f 13.5; plates, Wellington spectrum panchromatic plates; time of exposure, 2.5 min.; developer, Ilford metol developer for soft effects; time of development, three minutes.

The light filter was chosen for the region of the spectrum where a solution of carbonmonoxide-hemoglobin has its two strong absorption bands. By this precaution the effect of traces of met-hemoglobin formed in the solution was eliminated.[14]

x_1	x	c_1	c_2	$M \times 10^{-3}$	$M/16.700$
in cm.		in g. per 100 cc.			
4.61	4.56	1.220	1.061	71.30	4.27
4.56	4.51	1.061	0.930	67.67	4.05
4.51	4.46	0.930	.832	58.33	3.49

[13] These measurements should be regarded more as an illustration of the method than as a precision determination of the molecular weight of hemoglobin. A more refined technique of measurement will, we hope, enable us to communicate such determinations later on.

[14] In this and the following experiments the bottom of the cell was covered with a layer of pure mercury in order to give to the column of solution a peripheral boundary surface of exactly the right curvature.

TABLE I (*Continued*)

x_1	x	c_1	c_2	$M \times 10^{-3}$	$M/16.700$
in cm.		in g. per 100 cc.			
4.46	4.41	.832	.732	67.22	4.02
4.41	4.36	.732	.639	72.95	4.37
4.36	4.31	.639	.564	60.99	3.65
4.31	4.26	.564	.496	76.57	4.59
4.26	4.21	.496	.437	69.42	4.16
4.21	4.16	.437	.388	66.40	3.98
				Av. 67.87	Av. 4.06

2. Solution containing 1.0 g. of carbonmonoxide-hemoglobin and a 0.1 g. of sodium chloride per 100 cc. at the start; conductivity, 1.8×10^{-3} mhos in 2.0% solution; $V = 0.749$; length of column of solution, 0.55 cm.; thickness of column of solution, 0.145 cm.; speed, 7350 r.p.m. ($\omega = 245.0\pi$); $T = 292.6°$; time of centrifuging, 36 hours; lens, light source, filter, plate and exposure as in Expt. 1.

Curves traced from the microphotometric records are given in Fig. 5, the curve representing the relation between concentration and the ordinate of the microphotometric curves is reproduced in Fig. 6 and the curve giving the relation between concentration and distance from the center of rotation after sedimentation equilibrium has been reached is shown in Fig. 7.

4.76	4.71	1.315	1.193	68.05	4.08
4.71	4.66	1.193	1.080	70.29	4.21
4.66	4.61	1.080	0.984	66.45	3.98
4.61	4.56	0.984	.895	68.45	4.10
4.56	4.51	.895	.806	76.44	4.58
4.51	4.46	.806	.729	74.13	4.44
4.46	4.41	.729	.661	73.10	4.38
4.41	4.36	.661	.601	71.87	4.30
4.36	4.31	.601	.548	70.53	4.22
4.31	4.26	.548	.500	70.86	4.24
				Av. 71.02	Av. 4.25

3. Met-hemoglobin solution containing 1.0 g. per 100 cc. at the start; conductivity, 1.1×10^{-4} mhos in a 2.0% solution; $V = 0.747$; length of column of solution, 0.6 cm.; thickness of column of solution, 0.145 cm.; speed, 7180 r.p.m. ($\omega = 239.3\pi$); $T = 290.2°$; time of centrifuging, 40 hours; light source, incandescent lamp with white bulb and no light filter; aperture of photographic lens, f 22.5; plates, Imperial Eclipse; time of exposure, 0.5 minutes; development as in Expt. 1.

4.65	4.60	1.335	1.211	71.18	4.26
4.60	4.55	1.211	1.109	64.94	3.89
4.55	4.50	1.109	1.007	71.99	4.31
4.50	4.45	1.007	0.926	63.28	3.79
4.45	4.40	0.926	.838	76.21	4.56
4.40	4.35	.838	.764	71.36	4.27
4.35	4.30	.764	.697	71.66	4.29
4.30	4.25	.697	.640	67.39	4.04
				Av. 69.75	Av. 4.18

4. Met-hemoglobin solution containing 1.0 g. per 100 cc. at the start; conductivity, 3.1×10^{-4} mhos in a 2.0% solution (electro-dialyzed); $V = 0.747$; length of column of solution, 0.55 cm.; thickness of column of solution, 0.145 cm.; speed, 7240 r.p.m. ($\omega = 241.3\pi$); $T = 293.2°$; time of centrifuging, 36 hours; aperture of lens, light source and plates as in Expt. 3.

4.87	4.82	1.473	1.342	64.32	3.85
4.82	4.77	1.342	1.244	53.22	3.19

TABLE I (*Concluded*)

x_1	x	c_1	c_2	$M \times 10^{-3}$	$M/16.700$
in cm.		in g. per 100 cc.			
4.77	4.72	1.244	1.138	63.04	3.78
4.72	4.67	1.138	1.037	66.48	3.98
4.67	4.62	1.037	0.953	60.64	3.63
4.62	4.57	.953	.875	62.71	3.76
4.57	4.52	.875	.806	60.61	3.65
4.52	4.47	.806	.740	63.66	3.81
4.47	4.42	.740	.676	68.02	4.07
4.42	4.37	.676	.622	63.28	3.79
4.37	4.32	.622	.576	59.52	3.56
				Av. 62.32	Av. 3.73

in favor of the view that at least in one and the same solution there is practically only one kind of hemoglobin molecule. It is the fact that in none of the series of determinations hitherto made has there been observed any systematic variation of molecular weight with distance from the center of rotation. If there were molecules of different mass present in the same solution the values of the molecular weight calculated from measurements of the central part of the centrifuged solution should be lower than those calculated from measurements of the peripheral part. This is not the case.

The expenses connected with these experiments have been defrayed by a grant from the foundation "Therese och Johan Anderssons Minne."

Summary

1. A method has been described for the determination of the molecular weight of proteins based upon the measurement of sedimentation equilibrium in a protein solution exposed to a centrifugal force.

2. As an illustration of the method preliminary measurements of the molecular weight of carbonmonoxide-hemoglobin and of met-hemoglobin are given. The determinations indicate that hemoglobin solutions are built up of molecules containing four groups of molecular weight 16,700 that is, that the molecular weight of hemoglobin in aqueous solution is probably 66,800.

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